Franco SC, Augustin CB, Geffen AJ, Dinis MT.

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***Aquaculture* 2017, 468(Part 1), 569–578**

GROWTH, EGG PRODUCTION AND HATCHING SUCCESS OF *ACARTIA TONSA* CULTURED AT HIGH DENSITIES

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For the published version please refer to:

http://dx.doi.org/10.1016/j.aquaculture.2016.10.044

Franco S.C., Augustin, C., Geffen, A.J., Dinis M.T. (2017) Growth, egg production and hatching success of *Acartia tonsa* cultured at high densities. *Aquaculture*, 468:569-578.

*Abstrac*t

Acartia tonsa is a calanoid copepod with high potential as live feed for marine aquaculture. However, its usage remains limited at an industrial scale, with cost effective production being conditional on successful culture at high density. The present study took an integrated approach to provide further insight on the effects of *A. tonsa* stocking density on copepod growth and adult reproduction, specifically egg production and egg hatching success. The effect of stocking density was studied by following the growth and survival of *A. tonsa* copepods, from egg hatching to maturity, on cultures initially stocked with 250, 400, 1000, 3000 and 6000 copepods l^{-1}. Additionally, the effects of high-density rearing, of adults kept at 100, 250, 500 and 2500 copepods l^{-1}, on egg production and hatching success were also evaluated over a 5-day period.

Higher stocking densities were shown to have no unfavourable effect on copepod growth, though mortality significantly increased with density, from ≤ 2.5 % d^{-1} at densities of ≤ 1000 copepods l^{-1} to 3.5 - 4.0 % d^{-1} at 3000-6000 copepods l^{-1}. Individual egg production decreased with increasing stocking densities, from 28 eggs $female^{-1}$ d^{-1} at 100 copepods l^{-1} to 7 eggs $female^{-1}$ d^{-1} at 2500 copepods l^{-1}. However, total yield still increased with stocking density, with the cumulative egg production at 2500 copepods l^{-1} being 4 times the production at lower densities (from 100 - 500 copepods l^{-1}). Though adult rearing density had no effect on 96-h hatching rate (60 to 69 %), a density-dependent late hatching was observed on eggs produced by adults grown in dense cultures (500 - 2500 copepods l^{-1}), with 48-h hatching success significantly decreasing with increasing densities (from 37 % to 1 %).

In spite of the negative effect of stocking density on survival during growth and egg production, the magnitude of these effects does not compromise the use of high density cultures. Future research should focus on the improvement of production systems, as the ability to rear calanoid copepod species at large scale would present a major advancement in larviculture of marine fish species.

Keywords: copepod; culture; density; egg production

1. INTRODUCTION

Hatchery production of fish, molluscs and crustaceans has become of central importance to marine aquaculture and the supply of suitable food for early larval stages is one of the major difficulties during larval rearing, in particular with very small larvae. Current industrial larviculture relies to a great extent on diets of rotifers and brine shrimp for early feeding (e.g. Rainuzzo et al., 1997; Sargent et al., 1997), which besides being of concern in terms of nutritional suitability (e.g. Barclay and Zeller, 1996) and availability (Bengtson, 2003; Lavens and Sorgeloos, 2000) often constitutes a high fraction of the total production costs (e.g. People Le Ruyet et al., 1993; Conceição et al., 2010).

Copepods are a natural prey for the first-feeding of many marine fish larvae in the wild (Hunter, 1981; Pepin and Penney 1997; Støttrup, 2003) and the nutritional superiority over traditional live food is well established (Drillet et al., 2006a). Their use has been shown to lead to enhanced survival, higher growth and weight gain, decreased malpigmentation, higher rates of successful metamorphosis, increased stress resistance and increased feeding response in a number of species (e.g. Doi et al., 1997; Evjemo et al., 2003; Hernandez Molejon and Alvarez-Lajonchere 2003; Kraul et al., 1992; Luizi et al., 1999; Kuhlmann et al., 1981;; Shields et al., 1999). These and other attributes have increased the interest in large-scale culturing of copepods and recent literature has thoroughly discussed culturing techniques and the use of copepods as live prey in marine fish aquaculture (Conceição et al., 2010; McKinnon et al., 2003; Støttrup, 2000, 2003). However, copepod culture for industrial use is mostly done by *in situ* harvesting or semi-extensive outdoor growth (e.g. Engell-Sørensen et al., 2004; Toledo et al., 1999) and large scale culture is not widespread, in spite of the few published production protocols available for a small number of species (Abate et al., 2015; Payne and Rippingale, 2001; Schipp et al., 1999; Sun and Fleeger, 1995).

Acartia tonsa (Dana) is a calanoid copepod that is easily maintained in culture, though its usage in hatcheries remains limited, mainly due to difficulties in attaining reliable and cost efficient production at

an industrial scale, associated with a series of technical and biological challenges (Drillet et al., 2011). Extensive efforts have been done towards investigating the differences in the quality of produced eggs, storage effects and the analysis of differences between strains (Drillet et al., 2006a, 2006b, 2008a, 2008b; Hagemann et al., 2016; Hammervold et al., 2015; Hansen et al., 2016; Ohs et al., 2009). Literature has further centred its attention in identifying the main factors influencing *A. tonsa*'s culture performance, with focus on the effects of temperature and diet (Broglio et al., 2003; Kiørboe et al., 1985; Leandro et al., 2006; Støttrup and Jensen, 1990; Zhang et al., 2013, 2014), while photoperiod (Peck and Holste, 2006; Peck et al., 2008), salinity (Holste and Peck, 2006; Peck and Holste, 2006) and density (Drillet et al., 2014a; Jepsen et al., 2007; Medina and Barata, 2004; Peck and Holste, 2006; Zhang et al., 2014) have received less attention. Recent studies have addressed the economic feasibility of production and technical improvements to culture (Abate et al., 2015; Alver et al., 2011; Vu et al., 2014) and focused on gathering existing knowledge towards production (Drillet et al., 2011).

One of the main concerns is the success of production at high-densities (Drillet et al., 2011, 2014a; Støttrup, 2003), as increased copepod density can often decrease water quality and feed availability, while increasing conspecific interactions (Drillet et al., 2014a; Jepsen et al., 2013; Støttrup and Norsker, 1997). For *Acartia* sp., stocking density has been shown to affect not only growth (Medina and Barata, 2004), but also reproduction, namely egg production (Drillet et al., 2014a; Peck and Holste, 2006) and egg hatching (Camus and Zeng, 2009). For *A. tonsa* cultures, 50 to 100 copepods l^{-1} is considered standard stocking density, though a few studies have investigated the possibility of using higher densities (50 – 400 copepods l^{-1}, Peck and Holste, 2006; 100 – 600 copepods l^{-1}, Jepsen et al., 2007; 500 – 2000 copepods l^{-1}, Medina and Barata, 2004; 10 – 5062 copepods l^{-1}, Drillet et al., 2014a) either for copepod growth or egg production, with small scale experiments using densities as high as 6000 adults l^{-1} (Abate et al., 2015; Drillet et al., 2006b, 2008a). In spite of no reported effects of density on sex ratio (Jepsen et al., 2007; Medina and Barata, 2004) and mortality (Drillet et al., 2014a; Jepsen et al., 2007; Medina and Barata, 2004), negative effects were observed on development time (Medina and Barata, 2004), with conflicting results for egg production and hatching success (Drillet et al., 2014a; Jepsen et al., 2007; Peck and Holste, 2006). While Drillet et al. (2014a) only reported a decrease in the theoretical egg production per female above 2500 copepods l^{-1} (in spite of testing up to 5062 copepods l^{-1}), others reported this effect at much lower densities, of 191 – 600 copepods l^{-1} (nominal densities of 500 to 2000 copepods l^{-1}; Medina and Barata, 2004) and 65 to 425 copepods l^{-1} (Peck and Holste, 2006). Further to this, Peck and Holste (2006) and Jepsen et al. (2007) observed no differences in 48-h hatching success at densities under 600 copepods l^{-1}, while Drillet et al. (2014a) recorded a significant, though low reduction (1.7% per 1000 copepods) in 72-h hatching success. This variation in densities tested and the disparity between results, likely caused by differences in design and strains used (see Drillet et al., 2014a), further complicates the development of intensive systems. Furthermore, the efforts in investigating the effects of density on copepod growth and reproduction have often been decoupled, with most studies focusing solely on egg production and quality (e.g. Jepsen et al., 2007; Peck and Holste, 2006), but not on high-density growth followed by egg production (e.g. Drillet et al., 2014a).

A clear study of the performance of *A. tonsa* cultured at high-density, from copepod growth to egg production and hatching (see Medina and Barata, 2004, for ecotoxicological studies) is therefore essential to inform production using intensive systems. The present study evaluated the effects of stocking density (250 to 6000 copepods l^{-1}) on the development and survival of *A. tonsa*, from egg hatching to maturity, and continued by testing the effects of high-density rearing (100 to 2500 copepods l^{-1}) on egg production and hatching success for a 5-day period.

2. MATERIAL AND METHODS

2.1 *ACARTIA TONSA* STOCK CULTURES

Cultures from *A. tonsa* were started from eggs donated by the Danish Institute for Fisheries and Marine Research (now DTU-Aqua, Charlottenlund, Denmark), belonging to the Danish Sound population (DIFRES strain, DFH-ATI). This strain has been reared for over 70 generations in the laboratory, under

constant temperature and light conditions (salinity of 34, 17°C, dim light), on a diet of *Rhodomonas salina* (Drillet et al., 2008ab; Peck and Holste, 2006; Støttrup et al., 1986).

From the first mixed batch of copepods received (i.e. copepods from different cohorts and life stages), eggs were collected and several new stock cultures were established. The eggs were incubated and the hatched nauplii were cultured to adulthood, repeating the process for several generations. The cultures were kept in 10 to 80l polyethylene tanks, in UV-treated and 1µm-filtered seawater (salinity of 33), at 18 ± 1°C and 24:0 L/D photoperiod in dim light. Cultures were gently aerated and fed daily with *Rhodomonas baltica* and *Isochrysis galbana* (≥ 50.000 cells ml^{-1} day^{-1}; 1:1). Algal cultures were maintained in 10 and 30 l cultures, in bags, grown in UV-treated and 1µm-filtered seawater (salinity of 33) with addition of f/2 media (Guillard, 1975) at 18 ± 1°C and 24:0 L/D photoperiod, and were harvested at their exponential phase. Water quality was measured regularly, with partial daily water exchanges to assure good culture conditions (≥ 7.0 mg O_2 l^{-1} and pH 7-8). *A. tonsa* cultures were kept at low density (maximum 300 copepods l^{-1}) and were run for maximum period of a month until being discarded. Egg collection was done daily by removing aeration, allowing time for eggs to settle and attracting the copepods to the surface with light, while gently siphoning the settled eggs from the bottom of the tank. A double sieving system (150 µm / 50 µm) was used to separate adult copepods from eggs, which were rinsed in sea-water. The eggs were sampled, briefly rinsed in fresh water, covered with Parafilm, excluding air, and stored in 15 ml dark vials (≤ 50.000 eggs ml^{-1}) at 4 °C, for use in the later experiments.

2.2 Experimental design

2.2.1 Effects of stocking density on copepod growth and survival

The effect of stocking density (250, 400, 1000, 3000 and 6000 copepods l^{-1}) on the growth and survival of *A. tonsa* copepods was followed from egg hatching to maturity (February to April 2009). Due to technical limitations, the experimental design was divided in two experiments, I (12 days) and II (18 days), which tested, respectively, lower initial stocking densities of 250, 400 and 1000 copepods l^{-1} and higher densities of 1000, 3000 and 6000 copepods l^{-1} (3 replicates set per density treatment). The density of 1000copepods l^{-1} was included in both runs to establish the comparability of results between experiments I and II.

Cold stored eggs (maximum of 10 days old; from different females) were thoroughly mixed and incubated at 16 ± 1°C in 9l polyethylene tanks and otherwise cultured as described above. Cultures were fed daily with *Rhodomonas baltica* in excess to assure saturated food conditions (≥ 1000 µg C l^{-1}), calculated after measurement of algal volume (Multisizer™ 3 Coulter Counter, Beckman Coulter) and conversion to carbon content (44 pgC $cell^{-1}$), following Strathmann (1967). Algal density was monitored daily pre-feeding by means of direct counting using a Neubauer chamber and feed concentration was adjusted by adding fresh algae (≤ 200 mL) harvested at the exponential phase. Initial algal content per replicate was therefore estimated from the sum of the algae added and algae concentration sampled pre-feeding. The water volume of 9L was checked daily and readjusted as necessary, to account for volume losses by sampling and evaporation and additions from feeding, to assure a constant culture volume. Water was exchanged every 2 to 3 days, monitored daily for temperature and oxygen levels (WTW Oxi 330i Meter), and bi-weekly for salinity and pH (WTW ProfiLine Multi 1970i Portable Multiparameter Meter) to assure suitable culture conditions. Daily sampling consisted of sieving 200 ml of culture volume twice with a 45 µm sieve to isolate the copepods. Afterwards the copepods were rinsed with seawater into 15 ml falcon tubes, preserved with Lugol solution and stored as samples in dark conditions until being analyzed. Each sample was photographed (Nikon digital sight DS-Fi1 Camera). Afterwards copepods were counted, measured for total length (TL, µm) and identified according to development stage and sex (according to Bradford-Grieve, 1999; Sabatini, 1990; Ogilvie, 1953). Measurements were made with Image J (1.46r; Schneider et al. 2012) for total body length of nauplii (BL, µm) or total prosome length in copepodites and adults (PL, µm). Copepod dry mass (DM, µg) was calculated for nauplii and copepodites, from respectively BL and PL, following Berggreen (1988) and used for the calculation of mass-specific growth rates (SGR, as the ln mass gain per day). Median development time (MDT, days) was estimated from stage frequency data, considering the time required for 50 % of

copepods to reach the last development stage (see Campbell et al., 2001, Landry, 1975). Population related parameters such as stocking density (copepods l^{-1}), daily mortality (% d^{-1}) and sex ratio (shown as % of females) were calculated directly from copepod counts. The extractive sampling procedure caused a sampling-induced mortality across treatments and therefore the corrected mortality has been calculated by applying a negative interest rate, which accounts for the daily sampling of 2.22% of the culture volume.

2.2.2 Effects of stocking density on egg production and egg hatching success

The effect of stocking density (100, 250, 500 and 2500 copepods l^{-1}) used during the culture of copepods to maturity, on egg production of *A. tonsa* adults was followed for 5 days (3 replicates per treatment) on Experiment III. The eggs produced by copepods raised at high densities were further left to hatch for 48 h and 96 h to evaluate the effects on the hatching success.

Pre-experiment, the eggs, from different females, were thoroughly mixed and incubated at 17 ± 1°C in 9 l polyethylene tanks under conditions as previously described, but 8:16 L/D photoperiod. At the start of development period (day 3), treatments had initial densities of 264 ± 35, 595 ± 57, 1133 ± 98 and 6034 ± 201 copepods l^{-1}. After 18 days of culture no other water changes were executed and copepods were left to acclimatize for 72 h until the beginning of the egg production experiment. Copepod density after 21 days of culture, at the end of the development period and start of the egg production experiment, consisted of 109 ± 23, 248 ± 44, 517 ± 77 and 2523 ± 238 copepods l^{-1}. These will hereafter be referred to as nominal densities of 100, 250, 500 and 2500 copepods l^{-1}, respectively.

Cultures were maintained for 5 days in the conditions previously described for *A. tonsa* stock cultures, while sampling took place. Daily sampling consisted of gentle mixing of the tank, and removing 200 ml of culture volume, collected after, which was sieved through a 45 µm plankton filter. Isolated copepods and eggs were rinsed and preserved as described earlier. All samples were photographed, with copepods counted and identified according to sex, to allow for the estimation of stocking density and sex ratio, as described. The eggs were collected daily by siphoning from the tank bottom, and incubated for 96 h (≥30 eggs $sample^{-1}$; 3 replicate samples) at 18 ± 1°C and 24:0 L/D (dim light). The number of dead eggs, unhatched eggs, hatched eggs and nauplii were counted at 0, 48 and 96 h, to allow for the calculation of hatching success (HS; % at 48-h or 96-h). Egg production was described by calculated parameters such as individual egg production (IEP, eggs $female^{-1}$ d^{-1}), relative egg production (REP, eggs l^{-1} d^{-1}), and total egg production (TEP, eggs $tank^{-1}$ d^{-1}).

2.3 Statistical analysis

All statistical analyses were performed using STATISTICA 7.0® and data in percentage (%) were arcsin transformed. Data were subjected to parametric tests as t-student test and analysis of variance (ANOVA), when assumptions for normality and homocedasticity were met (Shapiro-Wilk and Levene test, respectively), considering a significance level of α=0.05. Significant ANOVAs were followed by a Tukey-test to identify differences among groups. Data that did not meet the assumptions for normality and homocedasticity were subjected to non-parametric tests, namely the Kruskal-Wallis test. Analysis of density change over time was also conducted through simple linear regression. All figures and tables shown are represented with the mean ± standard error (se), except when mentioned otherwise, as all the values presented.

3. Results

3.1 Effects of stocking density on copepod mortality, growth and development

3.1.1 Density and mortality

Differences in culture stocking densities and daily copepod mortality are shown in **Fig. 1a** and **1b**, respectively. As sampling was extractive, a sampling-induced mortality was caused across treatments and therefore both original and corrected data are presented for clarity. Density decreased steadily over time

for all treatments (**Fig. 1a**), with higher total mortality in cultures run for longer periods, but with average daily mortality below 4 % for all densities (**Fig. 1b**). Daily mortality was not statistically different at densities below 1000 copepods l^{-1} (experiment I; original data: ANOVA, F=0.52, p value=0.60; corrected data: ANOVA, F=0.29, p value=0.76). In contrast, during experiment II, daily mortality was significantly different between cultures grown at different densities (experiment II; original data: ANOVA, F=5.61, p=0.02; corrected data: ANOVA, F=3.99, p value=0.04), suggesting a density-dependent mortality in cultures with stocking densities above 1000 copepods l^{-1}. Though in the original data, mortality was significantly different between cultures grown at 1000 cop l^{-1} and higher densities of 3000 and 6000 cop l^{-1} (Tukey test, p=0.01), these differences were less marked on the corrected data (shown by different letters in Fig. 1b) where only cultures grown at 1000 cop l^{-1} and 6000 cop l^{-1} showed significantly different mortalities (Tukey test, p=0.03). In the experiment II tests, original daily mortality was 2.54 ± 0.42 %, 3.55 ± 0.19 % and 4.00 ± 0.28 % respectively at 1000, 3000 and 6000 copepods l^{-1}. Corrected daily mortality was 1.35 ± 0.49 %, 2.47 ± 0.25 % and 3.07 ± 0.50 % respectively at 1000, 3000 and 6000 copepods l^{-1}. Although the eggs used in experiments I and II came from different batches, the mortality in the repeated treatment of 1000 copepods l^{-1} was not statistically different (ANOVA, F=1.60, p=0.19), supporting comparisons between the results from both experimental experiments I and II.

3.1.2 Growth and development

Copepod stage development over time and according to culture density is shown in **Fig. 2**. All cultures showed similar patterns in stage progression, with early copepodites (C I) appearing by day 8, late copepodites (C IV) by day 11/12 and maturity reached after 16 to 17 days in culture, except for experiment I which only lasted 12 days. The increase in copepod size over time is shown in **Fig. 3**. Average mass-specific growth rates were observed to change according to periods of prevailing nauplii (days 1 to 7; experiment I: 0.45 ± 0.03 ng C d^{-1}; experiment II: 0.47 ± 0.05 ng C d^{-1}), transition from nauplii to copepodites (days 8 to 10; experiment I: 0.33 ± 0.05 ng C d^{-1}; experiment II: 0.33 ± 0.07 ng C d^{-1}) and copepodite dominance (days 11 to 18; experiment II: 0.28 ± 0.04 ng C d^{-1}). There were no differences between treatments (experiment I: ANOVA; F=0.01, p=0.99; experiment II: ANOVA, F=0.09, p=0.91), as all cultures developed at similar rates and without observable delays in development (**Fig. 2**). Observed growth rates were 0.42 ± 0.03 ng C d^{-1} during experiment I (12 days) and 0.33 ± 0.22 ng C d^{-1} during experiment II (18 days). Nevertheless, in experiment II (longer running time) a non-significant difference in mass-specific growth rate (T-test: df=73; p=0.05) was observed between nauplii and copepodites, which grew on average 0.28 ± 0.01 d^{-1} and 0.17 ± 0.01 d^{-1}, respectively, without significant differences between treatments (nauplii: ANOVA, F=0.07; p=0.93; copepodites: ANOVA, F=0.23; p=0.80).

The co-occurrence of individuals in overlapping stages was noticeable over the whole culture period, but size distribution was similar among treatments, with no clear relation between density and increasingly mixed-stage cultures found. Stage development was independent of culture density and no differences were observed in the median development time (**Table 1**) among culture densities. Identical stages tended to be expressed in the different culture densities at similar times with an average difference of 3 – 6 h. Each stage lasted between 12 to 48 h until moulting to the next stage.

Water quality parameters are shown in **Table 2**, with no differences noted for any of the measured parameters. Dissolved oxygen was found to be on average over 9 mg l^{-1}, with saturation over 90 %, though a slight decrease to 75–80 % was generally noted after a week of culture, on the second and third day after each water change, after which when values would stabilize.

3.2 Effects of adult stocking density on egg production and egg hatching success

3.2.1 Adult copepod mortality and sex ratio

Copepod mortality was not significantly different between treatments (ANOVA, F=1.28; p=0.34; 2.31 ± 0.17 % d^{-1}). Similarly, in spite of the sampling-induced mortality during this period, no significant differences were observed for corrected mortality between treatments (ANOVA, F=0.82; p=0.52; 1.18 ±

0.30 % d^{-1}). Water quality parameters are shown in **Table 3**, with no observed differences noted for any of the measured parameters. Sex ratio tended most frequently to 2:1 F/M, with females constituting on average 57 % of the population (from 41 to 76 %, with no significant differences between treatments (ANOVA, F=1.36; p=0.27) or culture time (ANOVA, F=0.76; p=0.56).

3.2.2 Egg production

Average individual egg production (IEP, **Fig. 4a**) decreased slowly over time. From day 1 to day 5 (**Fig. 4b**) a significant decrease in IEP was noted for the densities of 100, 250 and 500 copepods l^{-1} (ANOVA, F=166.21; p<0.01; Tukey test: p<0.01). A similar pattern was noted for relative egg production (REP), which decreased significantly with time at 500 and 2500 copepods l^{-1} (Tukey test: p <0.03), dropping approximately 50 % over the experimental period. Adult stocking density significantly impacted IEP (**Fig. 5**), with all densities being significantly different (ANOVA, F=72.34; p<0.01; Tukey test: p<0.001). Though sampling-induced mortality might have affected IEP, the experimental time of 5 days limited the number of extracted females over time and therefore original data (herein presented) has a maximum overestimation of 1 egg $female^{-1}$ d^{-1} in comparison to corrected IEP (not shown). Individual egg production decreased from 28.4 $\pm$ 1.43 eggs $female^{-1}$ d^{-1} at 100 copepods l^{-1} to 7.13 ± 0.61 eggs $female^{-1}$ d^{-1} at 2500 copepods l^{-1}. However, REP increased significantly with stocking density (ANOVA, F=28.81, p<0.01; **Fig. 5**) and in spite of no significant differences at densities from 100 to 500 copepods l^{-1} (Tukey test: p≥0.23), REP was significantly higher at 2500 copepods l^{-1} (Tukey test: p<0.01). This pattern translated to similar differences in total egg production (**Fig. 6a**). Accumulated total egg production increased over the 5 days (**Fig. 6b**), with 89908 ± 7647 and 365538 ± 43176 eggs produced per tank after 5 days, for the experimental groups of 100 and 2500 copepods l^{-1} respectively.

3.2.3 Egg hatching success rate

Eggs collected on different days had similar hatching success (HS) rates (**Table 4**), with no effect of egg collection time on HS at either 48 h (ANOVA, F=1.88; p=0.13) or 96h (ANOVA, F=0.33; p=0.85).

Hatching success after 48h was significantly different between treatment groups (ANOVA, F=21.79; p<0.0001), decreasing with increasing adult rearing densities. Recorded 48-h HS was of 37.06 ± 5.11, 28.86 ± 2.70, 16.28 ± 2.69, and 1.23 ± 0.36 % for eggs produced by adults grown at 100, 250, 500 and 2500 copepods l^{-1} respectively (**Fig.7**). On the other hand, HS after 96 h was not significantly different between densities (ANOVA, F=0.91; p=0.46) and was above 60 % for all groups. For the experimental groups of 100, 250, 500 and 2500 copepods l^{-1}, 96-h HS was respectively 69.73 ± 4.69, 66.16 ± 6.10, 63.50 ± 3.13, and 60.33 ± 2.29 % (**Fig. 7**).

4. Discussion

4.1 Effects of stocking density on mortality, growth and development

4.1.1. Copepod mortality

Estimated daily mortality (0 to 4 % d^{-1}) is in the lower range of values reported in literature for mortality during development of *A. tonsa*, which range from 5 % d^{-1} (Støttrup et al., 1986) to 8 % d^{-1} (Medina and Barata, 2004). Nevertheless, the significant difference in mortality suggests a density-related mortality at densities above 1000 copepods l^{-1}, even when results are corrected for sampling-induced mortality, which could be related to the intensified conditions of overcrowding, increased accumulation of metabolites and higher competition for food and space (Sibly et al., 2000; Støttrup et al., 1986). Other factors, such as deteriorating water quality, can also be a determinant since calanoid copepods have low tolerance to poor water quality (Payne and Rippingale, 2001) and especially to the increase in ammonia concentration, non-nutritious suspended particulate matter (not monitored in the present study) and proliferation of contaminant species (Schipp et al., 1999). Density related mortality has been observed in adult cultures of *A. sinjiensis* (Camus and Zeng, 2009) grown from 100 to 600 copepods l^{-1}. However, in the only study that evaluated nauplii and copepodite density effects on *A. tonsa* survival, Medina and Barata (2004) reported no relation between the two factors, when testing densities from 500 to 2000 copepods l^{-1}. The results from the present study agree with the previous authors, showing no differences in mortality rates

under 1000 copepods l^{-1}, but present further data that shows that although density doesn't have an effect on mortality at densities equal or lower than 1000 copepods l^{-1}, there seems to be an increase in mortality with increasing densities up to 6000 copepods l^{-1}. Nevertheless, a 6 fold density increase, from 1000 to 6000 copepods l^{-1}, is translated into approximately a 2 fold decrease in survival, which might still constitute a profitable situation if the goal is to intensify copepod production.

4.1.2. COPEPOD GROWTH AND DEVELOPMENT

Development time agreed with previous literature (15-20 days at 16 – 18°C; Calbet and Alacraz, 1997; Støttrup et al., 1986; Leandro et al., 2006), with no observed effect of stocking density. However, Medina and Barata (2004) observed a delay in development in cultures grown at 2000 copepods l^{-1} (in comparison with 500 and 1000 copepods l^{-1}). This might be due to differences in culture conditions between studies, namely diet and temperature, and it cannot be excluded that a differential impact of sampling on culture fitness could have occurred in the previous study. Furthermore, stage progression in the present study was in agreement with APHA (1989) and growth rate showed no differences according to density, though they were in the lower range in comparison to previous studies (0.23 - 0.25 d^{-1} at 20°C, Heinle, 1969; 0.53 d^{-1} at 20 °C, Miller et al., 1977) likely due to the lower rearing temperature used. Though water quality and food quantity were closely monitored during the experiments, it cannot be excluded that other non-monitored factors might have affected growth (e.g. algal quality). The overall lack of significant differences between stocking densities and the non-limiting culture development is further corroborated by the fact that though differences between nauplii and copepodite stage growth rates were observed, as in Leandro et al. (2006) and Bergreen et al. (1988), these were not different between treatments. Moreover, isochronal development was also observed to be similar, independent of density, as in Klein Breteler et al. (1982), with stage duration ranging from 1 to 2 days, in agreement with Heinle (1969), who reported development rate at the population level to be 1.6 stages d^{-1} at 20ºC.

4.2 EFFECTS OF ADULT STOCKING DENSITY ON EGG PRODUCTION AND EGG HATCHING SUCCESS

4.2.1. EGG PRODUCTION

A. tonsa is known to produce an average of 25 to 50 eggs $female^{-1}$ d^{-1} at standard stocking densities of 50 to 100 copepods l^{-1} (Parish and Wilson, 1978; Kiørboe et al., 1985; Støttrup et al., 1986; Dam et al., 1994).). The fact that the maximum values obtained in the present study (33 - 39 eggs $female^{-1}$ d^{-1} at 100 copepods l^{-1}) are within the standard range supports that a satisfactory egg production was attained using this methodology. The gradual decline in egg production was observed for all density treatments (similar to Camus and Zeng, 2009; Jepsen et al., 2007; Peck and Holste, 2006 and unlike Drillet et al., 2014a), and suggest that the cultures were fully mature at the time of start of the experiment (21 days old, ≥ 90 % adults; see Parrish and Wilson, 1978) and had reached the level of maximum egg production.

Only a few studies have addressed the question of the effect of stocking density on egg production for *Acartia* spp. (**Table 5**), and most have stocked copepods at high-density at the time of measuring egg production rather than growing them for their full life cycle at high density (except Medina and Barata, 2004), and therefore comparisons are limited. Previous authors used densities of 500 to 2000 copepods l^{-1} during the growth phase, but when egg production started the densities were much lower (191 – 600 copepods l^{-1}), so it is unclear whether the effect observed was also valid at higher densities (e.g. Drillet al., 2014a). The present study considers the whole life cycle effects, and has therefore focused on the indirect effects of copepod stocking density during development to maturity on egg production and hatching success. The recorded values for individual egg production (IEP, 7 to 28 eggs $female^{-1}$ d^{-1}), which decreased with stocking density, were comparable with those reported in previous studies and further validate the results of Drillet et al. (2014a) up to 2500 copepods l^{-1} (actually 1963 copepods l^{-1} on average, over the 5-day period). This suggests that chemical and physical stress previously experienced by the adults at high-density might not have an added deleterious effect on egg production, in comparison to short-term stocking at high density.

In the present study, copepods grown from an initial stocking density of 6000 copepods l^{-1}, with 2500 copepods l^{-1} at the start of the experimental period, showed the lowest IEP. The inverse relation between stocking density and IEP, even in the absence of food limitation, has also been described by Peck and Holste (2006) in copepods stocked up to 425 copepods l^{-1} and by Drillet et al. (2014a) on densities up to 2500 copepods l^{-1}, above which IEP stabilized at minimum values and food became limiting. The increased contact between adults and subsequent behavioural and metabolic changes (Dur et al., 2011; Hansen et al., 1991; Razouls, 1972) as well as the deterioration of water quality (Payne and Rippingale, 2001) are known to be aggravated in high-density cultures (Gallucci and Ólafsson, 2007) and might contribute to the observed effect. Other factors, such as the increase in ammonia concentration and non-nutritious suspended particulate matter have also been shown to directly affect fecundity and egg viability in copepods (e.g. Buttino, 1994; Gasparini et al., 1999). On the other hand, cannibalism also occur at high density and the losses may be considerable, according to other studies (e.g. up to 16 % of *A. sinjiensis* nauplii mortality at 2000 copepods l^{-1}; Camus and Zeng, 2009), with Drillet et al. (2011, 2013, 2014b) recommending continuous egg harvest to improve yields. Increased competition for food or space as a result of overcrowding, may also significantly reduce food intake and have higher energetic costs as a result of increased swimming activity (Sibly et al., 2000; Støttrup et al., 1986). However, not all authors have reported detrimental density effects on egg production. Neither Jepsen et al. (2007), working with *A. tonsa*, nor Camus and Zeng (2009), investigating with *A. sinjiensis* observed any effect of density on egg production. The noted authors concluded that the discrepancies in effects between studies are probably species- or strain-specific for copepods. However, differences in methodology between studies are likely to lead to differences in results. One example comes from Jepsen et al. (2007), who reported a non-significant density-dependent effect and used small incubation chambers (250 ml) as a part of much larger tanks (60 l), therefore diluting the effect of chemical interactions and deterioration of water quality (Drillet et al., 2014), unlike the present study which attempted to approximate standard culture conditions.

In spite of the observed significant decrease in individual egg production, for relative/total egg production differences were only registered between the density of 2500 copepods l^{-1} and the remaining groups, with no differences between 100, 250 or 500 copepods l^{-1}. In fact, the observation that total accumulated egg production increased with stocking density has also been made by Peck and Holste (2006) and Jepsen et al. (2007) for *A. tonsa*, and Camus and Zeng (2009) for *A. sinjiensis*. However, Drillet et al. (2014a) observed a stabilization or decrease (depending on time) of relative egg production at densities over 1000 copepods l^{-1}, while Medina and Barata (2004) reported that the accumulated total egg production was higher at lower densities than at higher densities, which implied that there was no practical advantage in growing copepods at higher densities considering those results. Such an advantage would come from the decrease in IEP being lower than the increase in density, as it was verified here, where the total egg production at 2500 copepods l^{-1} was estimated to be approximately 4 times higher than egg production at 100 copepods l^{-1}. This indicates that, although it is less efficient to grow cultures at this density, since IEP declines drastically, in a situation of mass culture when space requirements are often limiting and feed costs are extremely relevant, high density cultures could be an alternative. A culture of 2500 copepods l^{-1}, producing the same number of eggs as a 100 copepods l^{-1} culture, would effectively occupy 4 times less volume. Drillet et al. (2014a) results must still be considered, as they caution against the use of higher densities if feed limitation is not resolved. Further to this, Drillet and Lombard's (2015) mathematical model shows that though losses by cannibalism generally increase with tank depth, up to 20% of eggs can still be lost by cannibalism in tanks of low depth (≤50 cm, as herein used). Since when considering cannibalism low depths also have the highest egg production per liter, especially at densities between 3000 to 5000 cop l^{-1} (Drillet and Lombard, 2015), care should be taken when extrapolating to larger industrial systems, where high-density production might in fact be lower. The development of improved culture systems, with automatic food control, separation of eggs from females and constant water renewal, will surely be key to minimize some of these issues.

4.2.2 Egg hatching success rate

Hatching success (HS) of eggs produced by adult copepods held at different densities showed a significant impact of density at 48-h of incubation, though no significant differences were detected among

treatments at 96-h of incubation, suggesting a delayed hatching. The indirect effect of copepod stocking density on egg HS of produced eggs has not previously been reported for *A. tonsa* and most studies have only reported the observation of HS at either 48 or 72h, but not both. Other high-density studies reported no effects on HS (e.g. 85 % HS at 48h, Jepsen et al., 2007; 45 % HS at 48 h, Peck and Holste, 2006), though these studies did not grow the adults at high density as in the current experiment, but only held them at high-density while monitoring egg production. The same was done by Drillet et al. (2014), where adults were stocked for monitoring egg production at high-densities and not grown under these conditions, though these authors did observe a slow decrease in 72-h HS with increasing densities. This was also correlated with higher number of dead eggs, but not to the delayed hatching eggs, unlike the present results.

Hatching success rates at 48-h were inversely related to stocking density, varying between 0 and 37 % at 2500 and 100 copepods l^{-1}, respectively, though after a further 48-h, all the groups showed similar densities, averaging 60 % HS. Though this effect has not been reported for *A. tonsa* before, similarities were found in *A. sinjiensis* eggs from adults cultured in higher densities also exhibited delayed hatching (Camus and Zeng, 2009). The previous authors suggest that this might have an evolutionary value in nature, preventing temporal overcrowding and reducing competition for food and space.

In copepods, low HS or hatching inhibition has been associated with the production of resting or non-viable eggs, female nutrition and diet fatty acid content, female age, female infertility or no mating, temperature, photoperiod among other factors (e.g. Marcus, 1980; Poulet et al., 1994; Jónasdottir and Kiørboe, 1996; Broglio et al. 2003; Dam and Lopes, 2003; Arendt et al., 2005). Peck et al. (2008) further refers to the significance of the conditions experienced by the adults (when the eggs were produced) and by the eggs (during their incubation; e.g. photoperiod), and relates low 48-h HS with increasing proportions of resting and of non-viable eggs produced. Ban and Minoda (1994) have shown that subjecting the cultures to high densities can also induce the production of resting eggs, due to the accumulation of metabolic products in the culture medium and lower water quality. Although this effect was not observed in this present study, the chemical effects of density could also partially explain the negative effect of stocking density on immediate HS. Drillet et al. (2008) described a pattern of low hatching in eggs with 0days of storage which increased after a determined period, likely related to the presence of eggs in delayed hatching stage, similar to the results observed in this present study. The fact that neither Peck and Holste (2006) or Jepsen et al. (2007) have reported an inverse effect of density on egg HS, might be explained by the fact that (1) the densities tested in these studies during egg production were lower, (2) feed and water quality might be aggravating factors in the present study influencing the female and/or egg condition, (3) previous studies did not grow the adults at high-densities, but only tested high densities during egg production. Additionally, the fact that Peck and Holste (2006) compared HS among groups with eggs collected on a single day and that Jepsen et al. (2007) used a system where adults were kept under permanent water renewal, may also explain the different results. Results from Drillet et al. (2014a), who observed a decrease in hatching with density, further support the first hypotheses, as these authors report a decrease in egg hatching with time at a rate of 1.7 % per every 1000 copepods l^{-1}, which would hardly be noticeable in lower density studies.

5. CONCLUSIONS

Higher culture stocking densities led to higher copepod mortality during growth, highlighting the need for careful consideration at an industrial scale over the benefits from increased space/resource optimization, against the disadvantage of copepod loss. On the other hand, the fact that increased densities did not delay or decrease copepod growth and development is of great advantage for commercial production of *A. tonsa*. Furthermore, in spite of the decrease individual egg production, egg yields were significantly higher at higher stocking densities, which suggests a great potential for the investigation of improved husbandry and system optimization in future studies. In addition, the observed effect of stocking density on hatching success indicates that longer incubation periods should be used to assure maximal hatching when growing broodstock at high density.

ACKNOWLEDGEMENTS

This work was partially supported by an ERASMUS grant (European Union) awarded to SF. The authors would also like to thank the Department of Biology, University of Bergen (UiB, Bergen, Norway) for hosting the research.

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681

TABLES

Table 1. Median development time (MDT, days) to each development stage (nauplii I to copepodite VI) according to initial stocking densities (experiment I: 250, 400 and 1000 copepods l^{-1}; experiment II: 1000, 3000 and 6000 copepods l^{-1}). Stages shown as *n.a.* were excluded from the analysis, since the period of development was considered to be incomplete and therefore MDT could not be calculated.

Development stage	250 cop l^{-1}	400 cop l^{-1}	*1000 cop l^{-1}	**1000 cop l^{-1}	3000 cop l^{-1}	6000 cop l^{-1}
Nauplii I	1.25	1.12	1.25	0.80	0.80	0.75
Nauplii II	2.50	2.60	2.60	2.40	2.50	2.75
Nauplii III	4.12	4.00	4.00	3.60	3.80	3.75
Nauplii IV	5.25	5.30	5.25	4.75	5.00	5.00
Nauplii V	6.00	6.10	6.12	5.30	5.50	5.75
Nauplii VI	6.60	6.60	6.50	7.00	6.50	6.40
Copepodite I	7.50	7.60	7.50	7.60	7.80	7.70
Copepodite II	9.50	9.10	9.12	9.50	9.10	9.20
Copepodite III	10.00	10.10	10.12	10.30	10.50	10.70
Copepodite IV	n/a	n/a	n/a	11.80	12.00	12.00
Copepodite V	n/a	n/a	n/a	13.70	13.50	13.25
Copepodite VI	n/a	n/a	n/a	15.25	14.75	14.60

* experiment I; ** experiment II.

Table 2. Water quality parameters (dissolved oxygen, mg l^{-1}; oxygen saturation, %; temperature, °C; salinity, psu; pH) during copepod development according to initial stocking densities (experiment I: 250, 400 and 1000 copepods l^{-1}; experiment II: 1000, 3000 and 6000 copepods l^{-1}). Initial feed quantity (µgC l^{-1}) refers to the estimated algae content available immediately upon feeding, while minimum feed quantity (µgC l^{-1}) was measured daily pre-feeding and refers to the lowest number of cells available at the time of sampling. Results are shown as mean ± se.

	250 cop l^{-1}	400 cop l^{-1}	*1000 cop l^{-1}	**1000 cop l^{-1}	3000 cop l^{-1}	6000 cop l^{-1}
Dissolved oxygen (mgL^{-1})	8.50 ± 0.59	8.51 ± 0.58	8.47 ± 0.51	8.95 ± 0.33	8.96 ± 0.32	8.95 ± 0.32
Oxygen saturation (%)	84.94 ± 5.66	84.90 ± 5.74	84.92 ± 5.62	89.33 ± 3.51	89.24 ± 3.23	90.37 ± 2.42
Temperature (°C)	16.08 ± 0.11	16.11 ± 0.10	16.10 ± 0.10	16.13 ± 0.11	16.11 ± 0.09	16.11 ± 0.11
Salinity (psu)	33.16 ± 0.06	33.16 ± 0.06	33.19 ± 0.07	33.55 ± 0.07	33.53 ± 0.09	33.55 ± 0.11
pH	7.64 ± 0.06	7.65 ± 0.07	7.66 ± 0.07	7.86 ± 0.03	7.87 ± 0.02	7.86 ± 0.03
Initial feed quantity (µgC L^{-1})	1162 ± 100	1324 ± 148	1380 ± 114	1324 ± 220	1597 ± 419	1882 ± 553
Minimum feed quantity (µgC L^{-1})	1126 ± 105	1259 ± 177	1306 ± 121	1114 ± 165	1175 ± 101	1196 ± 153

* experiment I; ** experiment II.

Table. 3 Water quality parameters (dissolved oxygen, mg l^{-1}; oxygen saturation, %; temperature, °C; salinity, psu; pH) and sex-ratio (% of females) during egg production experiment according to stocking densities (100, 250, 500 and 2500 copepods l^{-1}). Minimum feed quantity (µgC l^{-1}) was measured daily

immediately pre-feeding and refers to the lowest number of cells available at the time of sampling. Results are shown as mean ± se.

	100 cop l^{-1}	250 cop l^{-1}	500 cop l^{-1}	2500 cop l^{-1}
Dissolved oxygen (mg L^{-1})	9.09 ± 0.17	8.94 ± 0.27	8.99 ± 0.33	9.00 ± 0.19
Oxygen saturation (%)	90.08 ± 2.25	89.97 ± 3.10	89.12 ± 2.79	90.34 ±2.68
Temperature (°C)	16.16 ± 0.09	16.09 ± 0.12	16.12 ± 0.09	16.11 ± 0.12
Salinity (psu)	33.52 ± 0.08	33.56 ± 0.11	33.57 ± 0.08	33.53 ± 0.10
pH	7.86 ± 0.02	7.85 ± 0.02	7.86 ± 0.03	7.86 ± 0.04
Sex-ratio (% females)	59.04 ± 1.70	57.44 ± 1.99	54.60 ± 2.10	57.08 ± 1.64
Feed quantity (µgC L^{-1})	1406 ± 112	1436 ± 109	1396 ± 184	1355 ± 146

Table. 4 Hatching success (HS) rates after 48h and 96h (pooled data), during egg production experiment, according to experimental time (days). Results are shown as mean ± se.

	48-h HS	96-h HS
Day 1	26.77 ± 6.89	66.86 ± 2.62
Day 2	20.75 ± 4.70	58.21 ± 1.43
Day 3	18.15 ± 4.43	59.58 ± 3.82
Day 4	17.88 ± 3.61	67.70 ± 7.30
Day 5	17.67 ± 3.61	72.30 ± 4.50

Table 5. Summary of the literature on the effect of stocking density on the egg production of *Acartia* spp., according to the study, species (*A. tonsa* or *A. sinjiensis*), range of stocking densities tested (copepods l^{-1}), individual egg production recorded (IEP, eggs $female^{-1}$ d^{-1}) and whether significant density effects were found (Yes or No). Where differences were found, the average highest and lowest values of IEP correspond respectively to the lowest and highest densities tested, and where there are no differences the reported value corresponds to the average IEP. The table does not include reference studies (e.g. Støttrup et al., 1986) or studies run at high stocking densities of *Acartia* spp. but did not assess the effects of high-density growth (e.g. Schipp et al., 1999; Abate et al., 2015.

Species	Reference	Density (cop l^{-1})	IEP (eggs $female^{-1}$ d^{-1})	Significant differences
A. tonsa	Present study	100- 2500	7 - 28	Yes
	Drillet et al. (2014a)	10 - 5062	5 - 25	Yes
	Jepsen et al. (2007)	100 - 600	23	No
	Medina and Barata (2004)	191- 600*	5 - 16	Yes
	Peck and Holste (2006)	65 - 425	10 - 40	Yes
A. sinjiensis	Camus and Zeng (2009)	125- 2000	21	No

*nominal densities of 500 to 2000 copepods l^{-1}.

718 LIST OF FIGURES

719

720 **Fig. 1 (a)** Variation of density (copepods l^{-1}; mean ± se) over time (days), according to initial stocking
721 density (experiment I: 250, 400 and 1000 copepods l^{-1}, squares; experiment II: 1000, 3000 and 6000
722 copepods l^{-1}, circles), based on the original data. Corrected data, accounting for sampling-induced
723 mortality, is shown by linear trend lines, on experiment I for 250 copepods l^{-1} ($y=-1.10x+261.38$;
724 $R^2=0.04$; $F=0.38$; $p=0.55$), 400 copepods l^{-1} ($y=-0.77x+438.29$; $R^2=0.01$; $F=0.05$; $p=0.82$) and 1000
725 copepods l^{-1} ($y=-15.48x+1022.24$; $R^2=0.57$; $F=13.15$; $p<0.01$) and on experiment II for 1000 copepods l^{-1}
726 ($y=-24.73x+1084.45$; $R^2=0.64$; $F=28.77$; $p<0.01$), 3000 copepods l^{-1} ($y=-65.65x+3309.98$; $R^2=0.61$;
727 $F=25.13$; $p<0.01$) and 6000 copepods l^{-1} ($y=-118.82x+6356.37$; $R^2=0.53$; $F=17.99$; $p<0.01$). **(b)** Daily
728 mortality (% d^{-1}; mean ± se) according to initial stocking density (as previous) is shown according to both
729 original data (grey bars) and corrected data (black bars). Significant differences are indicated by different
730 letters for corrected data.

731 **Fig 2**. Percentage of copepods at each development stage (% n; left axis, colored bars) and density
732 (copepods l^{-1}; left axis; black curve) over time (days), according to initial stocking density, where (a) 250
733 copepods l^{-1}, (b) 400 copepods l^{-1} and (c) 1000 copepods l^{-1} (experiment I) and (d) 1000 copepods l^{-1}, (e)
734 3000 copepods l^{-1} and (f) 6000 copepods l^{-1} (experiment II). Different developmental stages were grouped
735 as eggs (eggs), nauplii (NI to VI), early stage copepodites (CI to III) and late stage copepodites (CIV to
736 CVI).

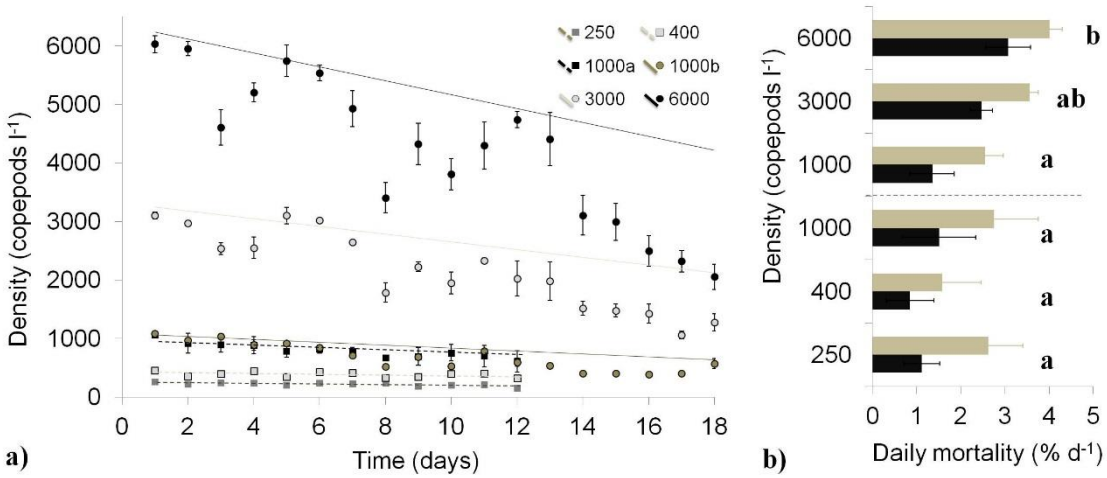
737 **Fig. 3** Average total length (TL, µm; mean ± se) of copepods in culture over time (days), according to
738 initial stocking density, for **(a)** experiment I: 250, 400 and 1000 copepods l^{-1} and **(b)** experiment II: 1000,
739 3000 and 6000 copepods l^{-1}).

740 **Fig. 4 (a)** Average individual egg production (eggs $female^{-1}$ day^{-1}; mean ± se) over time (days), shown
741 with power fit. **(b)** Individual egg production (eggs $female^{-1}$ day^{-1}; mean ± se) according to different
742 densities (100, 250, 500 and 2500 copepods l^{-1}) at the start (day 1; dark grey) and end (day 5; light grey)
743 of the experiment. Significant differences are indicated by different letters.

744 **Fig. 5** Individual egg production (IEP, eggs $female^{-1}$ day^{-1}; mean ±se; left axis) and relative production
745 (REP, eggs fl^{-1} day^{-1}; mean ±se; right axis) according to different densities (100, 250, 500 and 2500
746 copepods l^{-1}). Each set of points is fit with the respective power trend line.

747 **Fig. 6 (a)** Total egg production (thousands of eggs $tank^{-1}$ day^{-1}; mean ± se) according to different densities
748 (100, 250, 500 and 2500 copepods l^{-1}). Significant differences are indicated by different letters. **(b)**
749 Accumulated total egg production (thousands of eggs $tank^{-1}$) over time (days), according to stocking
750 density (100, 250, 500 and 2500 copepods l^{-1}).

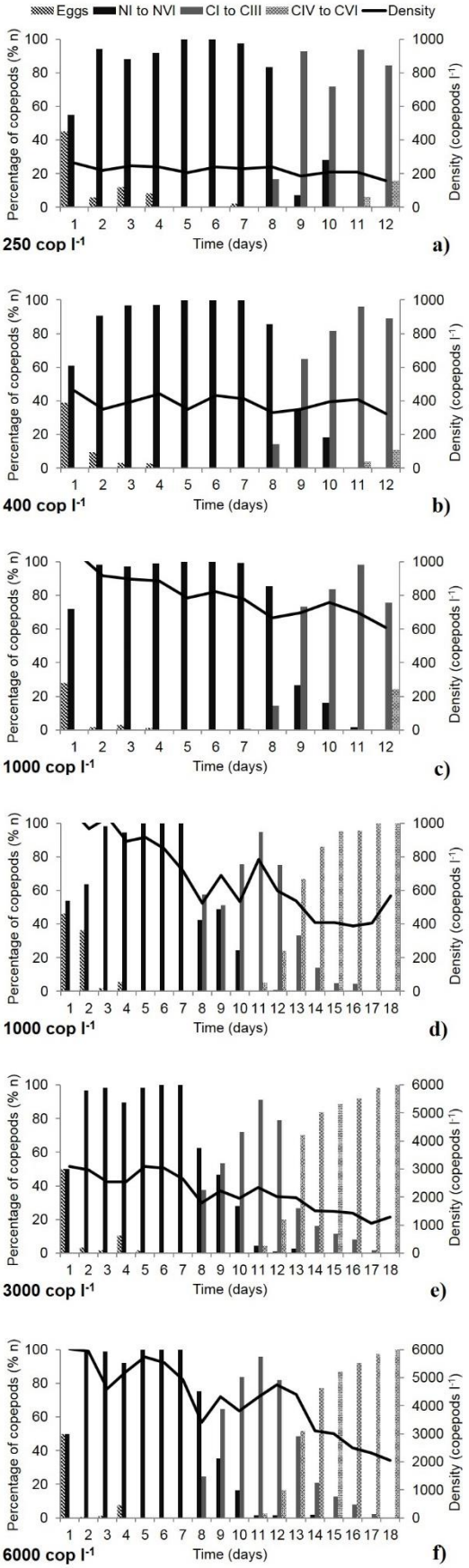
751 **Fig. 7** Egg hatching success (% of hatched eggs; mean ± se) at 48-h and 96-h, according to different
752 densities (100, 250, 500 and 2500 copepods l^{-1}) of parent copepods. Fitted curves are shown for 48-h and
753 96-h



754

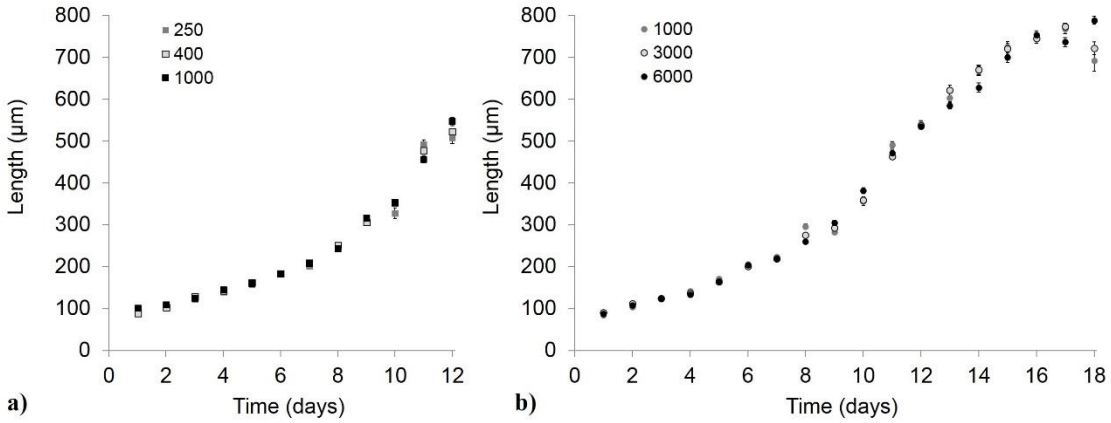
755 Fig.1

756


Eggs
NI to NVI
CI to CIII
CIV to CVI
Density
Percentage of copepods (% n)
Density (copepods l-1)
Time (days)
250 cop l-1
a)
400 cop l-1
b)
1000 cop l-1
c)
1000 cop l-1
d)
3000 cop l-1
e)
6000 cop l-1
f)

757

758 Fig.2



759

760 Fig.3

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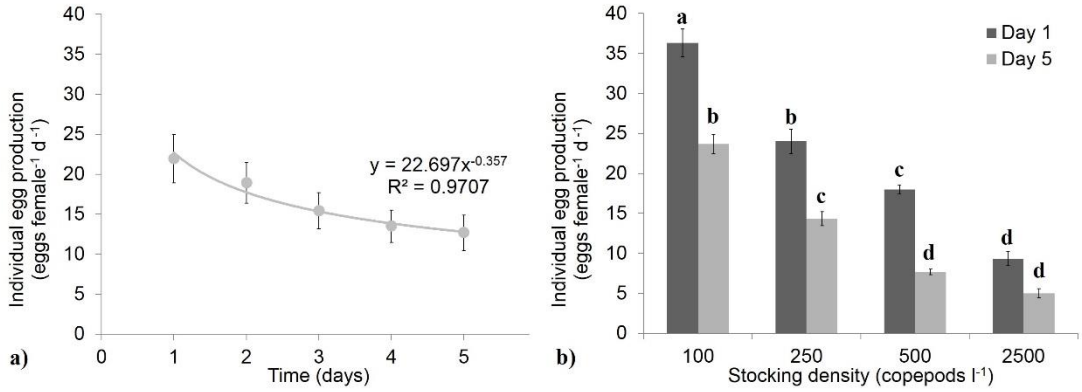
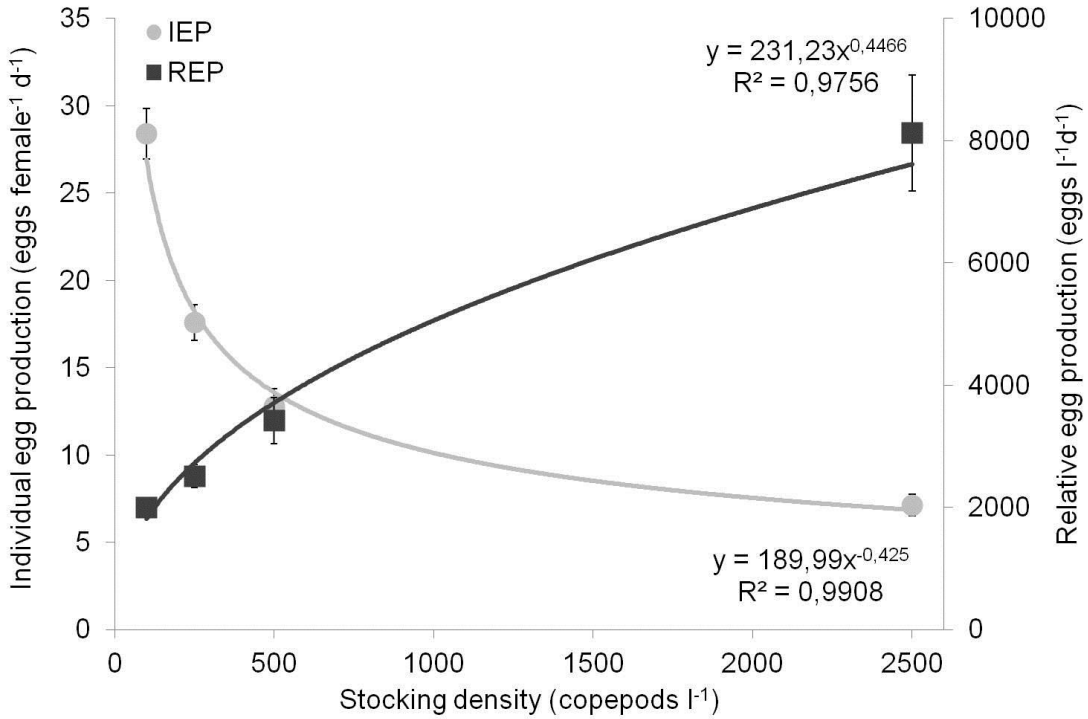


762

763 Fig.4

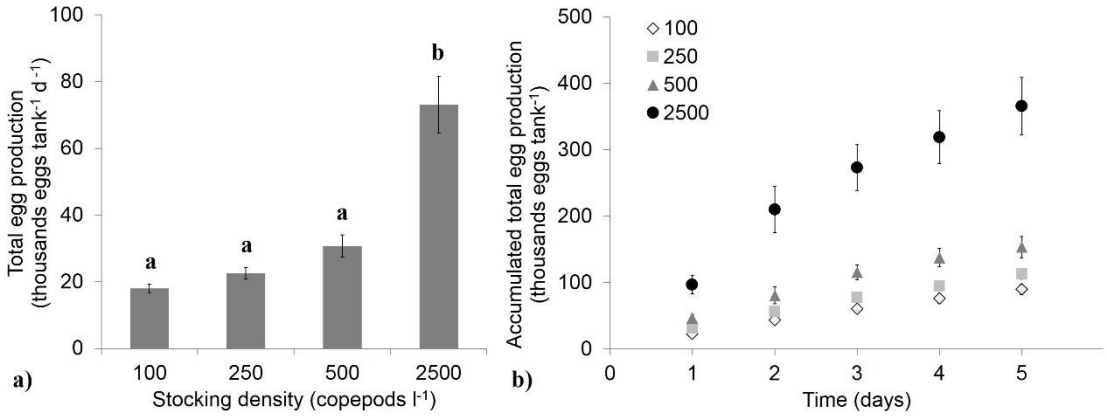
764



765

766 Fig. 5

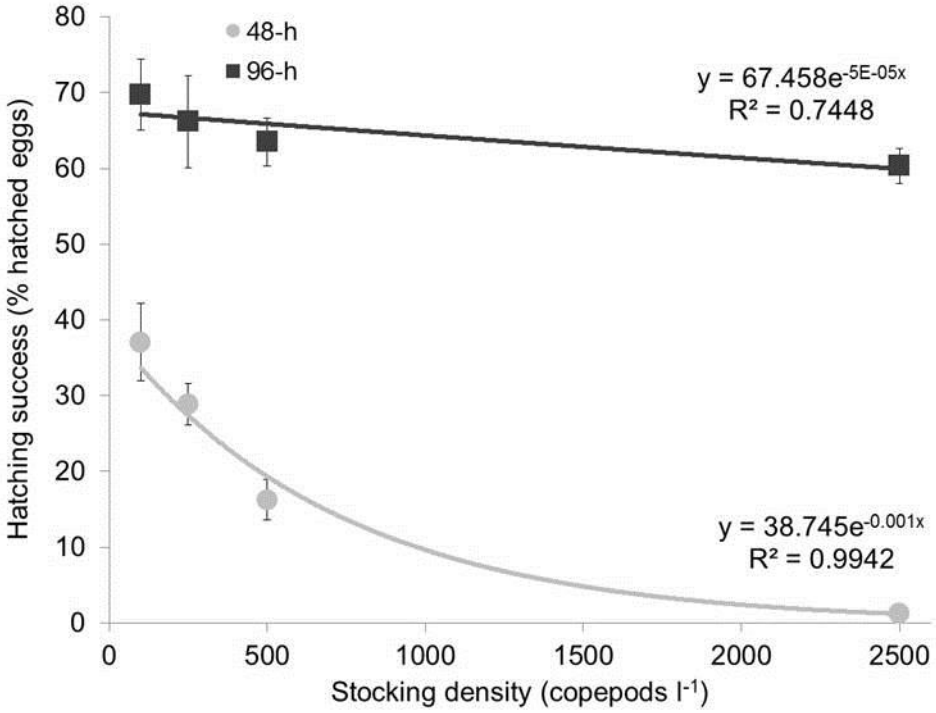
767



768

769 Fig. 6

770


48-h
96-h
y = 67.458e-5E-05x
R² = 0.7448
y = 38.745e-0.001x
R² = 0.9942
Hatching success (% hatched eggs)
Stocking density (copepods l-1)
0
10
20
30
40
50
60
70
80
0
500
1000
1500
2000
2500

771

772 Fig. 7